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The conversion of a normal cell into a cancer cell proceeds through a series of genetic and epigenetic alterations. We have proposed to use well-established genetic methodologies to identify novel anti-cancer targets via their specific, genetic interactions with common cancer mutations. In short, we will identify genetic alterations that are neutral in normal cells, but that are lethal when combined with cancer mutations. This "synthetic lethality" approach may identify potential therapeutic targets that are highly specific to the cancer cell.

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## Introduction

My laboratory has long been dedicated to the application of genetic principles to both the understanding of cancer biology and the search for novel anticancer targets. Since the 1970s, the war on cancer has been based on the notion that studying the disease will lead to the discovery of vulnerabilities, which can be exploited in the clinic. While many underlying genetic determinants of cancer have been identified, this knowledge has failed to translate into new therapeutic strategies, with only a handful of exceptions. One hypothesis is that this failure has largely been due to the genetically intractable nature of cultured mammalian cells. In part due to work from my laboratory, dsRNA-induced gene silencing, or RNA interference (RNAi), in mammalian systems has emerged as a tool that is likely to re-invigorate the field of somatic cell genetics and in the process revolutionize the study of human disease. During the past year, we have reached a point at which any gene in the human genome can conceivably be targeted using small dsRNA gene silencing triggers – *small interfering RNAs (siRNAs) or expressed short hairpin RNAs (shRNAs).* I have recently received an Innovator award from this same program that will fund the construction of shRNA-expression cassettes on a large, and possibly whole-genome scale. We will be able to use these tools within the present programs to test the notion that synthetic lethality is a plausible approach to the discovery of novel anticancer targets. I note that the goals of this grant and that of the Innovator award are distinct. The innovator award is to develop high-throughput procedures to create a resource for the entire cancer community. This program will apply that resource to a very specific problem in cancer biology.

## Introduction to the revised report

The original goal of this application was to develop strategies to permit synthetic lethal screens to be carried out in mammalian cells. In parallel, one needed to develop systems in which such interactions could be effectively tested. When this application was initially submitted four years ago, the technologies for searching for synthetic lethal interactions in mammalian cells were non-existent. During the funding period, my laboratory has played a major role in defining the biochemical mechanism of RNAi, and it is this work that has led to the ability to use RNAi as a tool in mammalian cells. In many ways, our efforts during this grant period have brought us to the brink of the ability to truly define a synthetic lethal interaction in a cancer cell. However, neither my group nor any other group has yet succeeded in meeting this goal.

Finding synthetic lethal interactions has as an absolute requirement the availability of a large resource that will permit the generation of a diversity of genetic lesions in mammalian cells. I would argue that – although trivialized by the reviewer in the comments on the initial version of this final report – the creation of an entirely new genetic technology that is now broadly applied in the field of cancer biology is somewhat more than activity or a milestone. I would

strongly argue that this is a research accomplishment that has had more of an impact on cancer biology than many of the outcomes that the reviewer of our initial report might classify as a more conventional research accomplishment. Nevertheless, it is fair to say that we have not yet identified a synthetic lethal interaction that can selectively impact the viability of a cancer cell. If the referee would deem the linked idea and cda award award a failure for that reason, so be it.

With regard to the comment that no real research results are described, I have included the list of kinases identified in the p53 screen. I have also included in the conclusions a discussion of the broader implications of the findings. As I have provided a list of the kinases, this report can no longer be considered for unlimited distribution as those results are still confidential.

I apologize for omitting publications from progress report from reportable outcomes. That these were not cited appropriately was my oversight and I apologize. I have now included these as reportable outcomes from the linked idea and cda award award.

Finally, I have expanded the report to include the entire period by including progress previously reported. I hope that you now find the report acceptable.

**Body**

*Genetically defined models of human cancer :*

One of the long-standing interests in our lab is the elucidation of the minimum genetic requirements for the transformation of human cells. Utilizing the technique of retroviral gene transduction, we have previously shown that normal human fibroblasts could be transformed with the oncogene combination of adenovirus E1A, MDM2, and H-RasV12. This transformation model is significantly different from those previously published in that there is a distinct absence of telomerase expression, either through direct introduction of the catalytic subunit hTERT or by an oncogene capable of activating hTERT. Analysis of telomerase status in these engineered cells and the resultant primary tumors indicates that telomerase is not activated, indicating that telomerase activity is not essential in the initial transformation event.

Upon explantation into culture, however, cells derived from these primary tumors undergo widespread apoptosis and senescence, phenomena indicative of telomere crisis, although a small percentage of explanted cells are able to survive this crisis event. Interestingly enough, the cells that survive after explantation are telomerase positive. When these cells are injected into a second nude mouse, the kinetics of tumor formation are similar to those of the primary tumors, indicating that no additional genetic alterations were acquired *in vivo* or *in vitro* other than telomerase activation. It is interesting to note that

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telomerase activation does not appear to enhance tumorigenicity, but serves as an enabling event to prevent telomere shortening and widespread genomic instability characteristic of cells undergoing mitotic crisis.

Through our studies utilizing this E1A-based system, we have begun to elucidate the minimum genetic alterations that must occur to transform a normal human cell into one that is cancerous.  Currently, we are focusing on taking this tractable transformation system and adapting it for use in an epithelial cell system to more accurately model events that lead to tumorigenesis.  In addition, we are also continuing to make progress on the development a human cell transformation model comprised entirely of cellular oncogenes.

This work was published as Seger et al., 2002.

*Loss of function genetics*

Over the several years, we have settled on the strategy of expressing short hairpin RNAs for doing genetic screens in mammalian cells.  After extensive comparisons of chemically synthesized siRNAs and shRNAs, we feel that shRNAs are at least as good, if not superior to, siRNAs.  In addition, shRNAs have the added benefit of being deliverable using a wide variety of reagents and well-worn strategies for gene transfer.  We have tested a number of strategies for shRNA expression (including different promoters, H1 and U6, different structural elements, different stem lengths etc.)  While these studies are still ongoing, we have settled on the U6 promoter and stem lengths of either 19 or 29 nt.  The decision between these two will be taken shortly and will be based on tests for off-target effects using microarrays.  This work was published as Paddison et al., 2002.

Furthermore, we have found that shRNA expression vectors are functional in living mice.  This provides a potential template for the use of RNAi as a gene therapeutic.   This work was published as McCaffrey et al., 2002.

We continue to develop strategies for delivering shRNA expression cassettes to cells in an efficient manner.  Specifically, we have tested several retroviral backbones, and have identified combinations of elements that can efficiently deliver functional shRNA expression cassettes into the genome (see figure 1).  Specifically, we have MoMuLV viruses and MSCV viruses that induce potent and stable suppression of target genes such as p16 INK4A and p53.
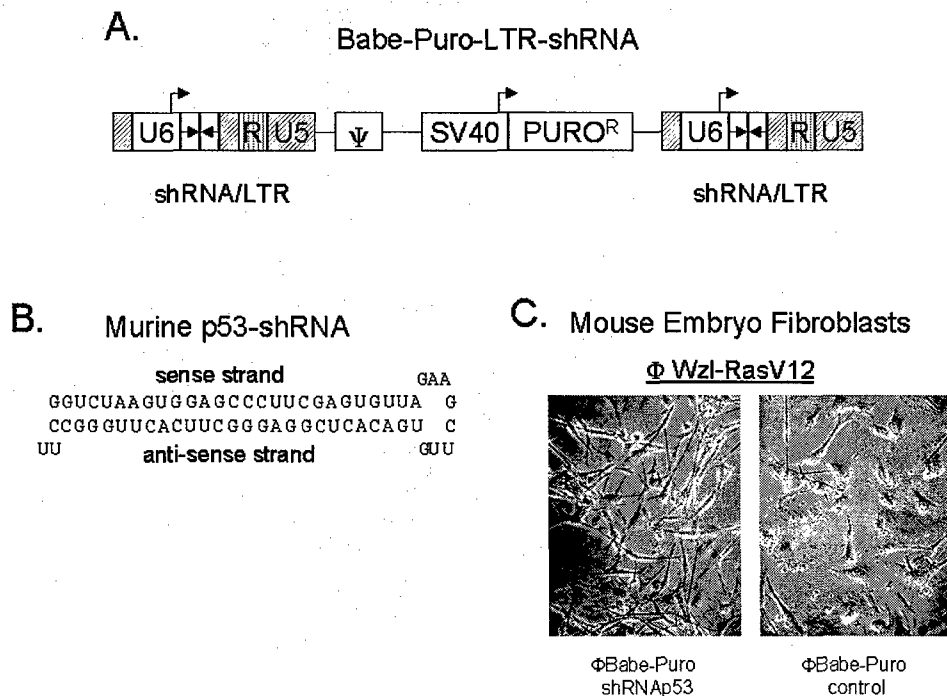
**A.** Babe-Puro-LTR-shRNA

shRNA/LTR                shRNA/LTR

**B.** Murine p53-shRNA

sense strand                    GAA
GGUCUAAGUGGAGCCCUUCGAGUGUUA  G
CCGGGUUCACUUCGGGAGGCUCACAGU  C
UU      anti-sense strand        GUU

**C.** Mouse Embryo Fibroblasts

Φ Wzl-RasV12

ΦBabe-Puro          ΦBabe-Puro
shRNAp53            control

**Figure 1: Stable expression of an shRNA using a retroviral system.** This figure demonstrates one strategy for expressing shRNAs from retroviruses. A U6-p53-shRNA was inserted into the 3' LTR of a MoMuLV pBabe-Puro retroviral construct. **A.** Shows the predicted structure and orientation of the retrovirus as integrated into the genome of the infected cell. **B.** The predicted structure of a murine p53 shRNA. **C.** An assay for bypass of rasV12 induced senescence in early passage mouse embryo fibroblasts (P2). Cells were first transduced with Babe-Puro alone or Babe-Puro-LTR-U6-p53-shRNA and selected in puromycin for 3 days, after which cells were infected with Wzl-Hygro-rasV12 and treated with hygromycin. Only cells initially receiving Babe-Puro-LTR-p53-shRNA were morphologically transformed by rasV12 and continued to divide (see text). A time point 5 days after transduction with rasV12 is shown. Potential complications with this strategy would arise if shRNAs target viral and drug resistance transcripts in packaging or target cells, or if shRNAs target essential genes in the packaging cells. Thereby we are currently designing inducible U6 constructs for expression from self-inactivating retroviruses.

## A Genome-wide shRNA library

Over the two years and with the support of an Innovator award, we have constructed a library of RNAi-inducing constructs that covers ~20,000 cancer relevant genes with from 1 to 6 shRNAs each (total to date of ~50,000 sequence-verified constructs). Additionally, last year, we constructed libraries consisting of ~20,000 mouse shRNAs targeting ~15,000 mouse genes. These libraries are the tools that are necessary to move forward with large-scale screens for synthetic lethality in mammalian cells. The library itself is being constructed with support from a number of sources (consider that the overall cost will be

~$9,000,000).  Since it is a key reagent for our studies, it is worthwhile to describe the resource here.

The first-generation library described in last year's report covered 10,000 selected genes.  This year our efforts have expanded in our second generation library to include all predicted genes in the human and mouse genomes.  These were constructed using optimized strategies both for shRNA expression and for bioinformatics predictions of effective shRNAs.

*shRNA optimization*

While our first-generation constructs were quite effective as suppressors of gene expression, last year saw the discovery of an additional step in the processing of small endogenous triggers of the RNAi machinery.  MicroRNAs produced from their natural genes are first transcribed by RNA polymerase II as long primary transcripts that are processed by a recently described complex (the Microprocessor) into pre-miRNAs.  It was the pre-miRNA structure that all first generation shRNA expression systems tried to mimic.  However, we have found that if we design our vectors to express a modified pri-miRNA structure based upon miR30, we can achieve approximately 10-fold higher expression of a small RNA in vivo than was achieved with the first generation vector systems (Figure 2).  We have therefore used this design in the construction of our second generation library.
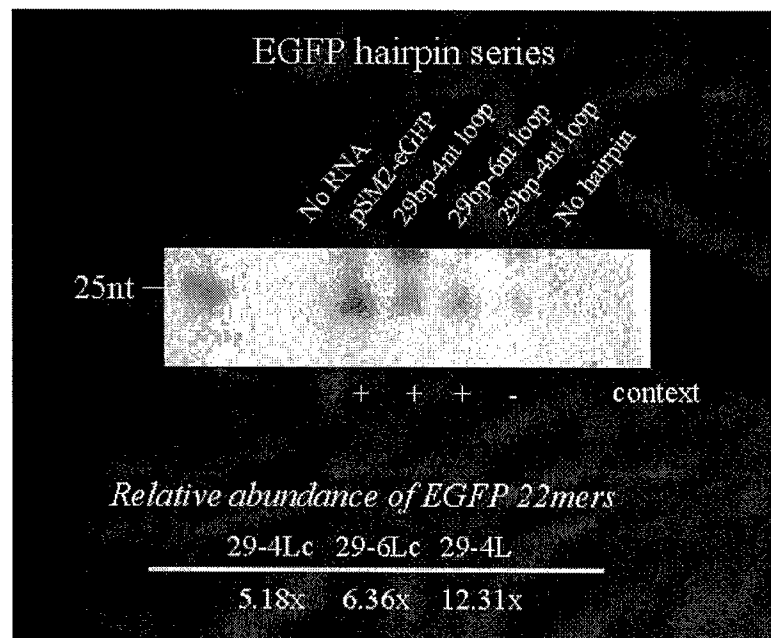


Figure 2.  pSM2, the second-generation shRNA vector, is compared to first generation vectors for small RNA production.  Specifically, 29bp-4nt loop lane shows the small RNA production from the first generation vector.

*shRNA library construction*

Using piezo-inject oligonucleotide synthesis, we have constructed second generation shRNA libraries that are now available to the academic community through Open Biosystems. As of this date, more than 500,000 vectors have been distributed from this source to the academic and commercial research communities. {An update for the revised report, this number stands at 1.2 million at present} This work was published as Cleary et al., 2004.


*Loss of function genetics*

The original goal of this grant was to use genetic technologies to enable loss-of-function approaches toward finding new anti-cancer targets in mammalian cells. The discovery that RNAi could be used as such a tool has been a tremendous boon. The availability of a large-scale RNAi-based library now makes our original goals possible.

We are taking two approaches toward identifying synthetic lethal interactions. The first is to use very standard approaches, using either morphological markers of cell death or fluorescent reports of caspase activity in vitro to identify lethal interactions. The second is to use highly-parallel approaches based upon the use of barcode arrays to identify such interactions.

One-by-one approaches

We have validated the second generation library in a screen for factors that modulate the activity of the p53 tumor suppressor pathway. To begin, we used a selected set of ~500 shRNAs that mainly target protein kinases. These were transfected into recipient cells individually in combination with reporter constructs that respond to p53 activation and with a construct that permits normalization for transfection efficiency. From this pilot study several kinases whose suppression leads to p53 activation were identified. Notably, when multiple shRNAs to the same gene were present in the collection, all the shRNAs to that gene gave the same phenotype. There were also instances in which we found both kinases and regulatory subunits of the same complex through our screen (figure 3). We are now studying how these kinases connect to the p53 pathway. Additionally, kinase inhibitors that activate p53 may present therapeutic opportunities with fewer side-effects than conventional p53 activators (e.g. DNA damaging agents). Even with the relatively limited screen that we have performed so far, several of the kinases that we have found are being evaluated as drug targets by Oncogene Sciences.
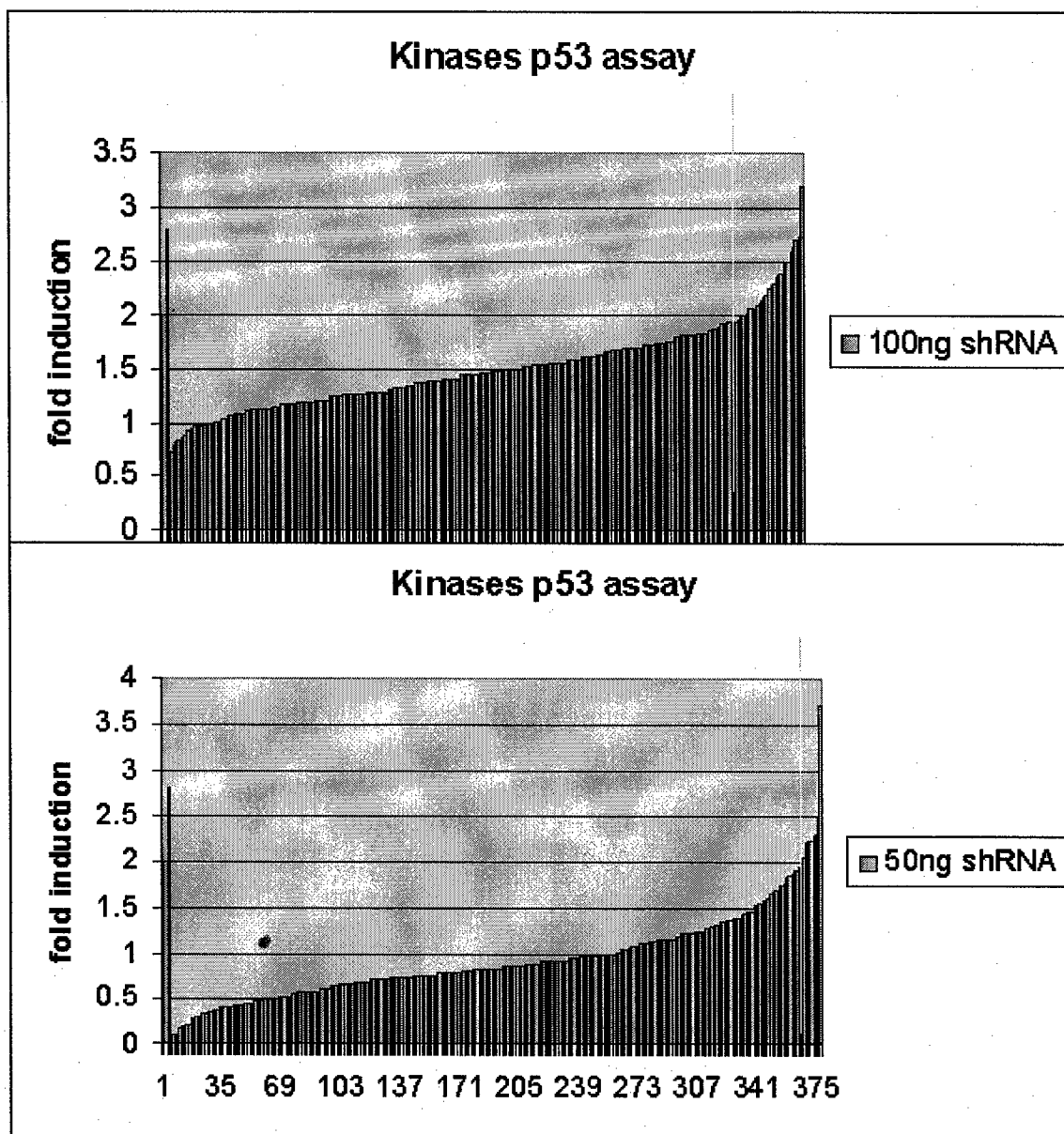
Figure 3. A screen of a partial human kinase collection for inducers of p53.
Positive signals to the left of each graph are shRNAs to mdm2 as controls.

This screen has identified a number of kinases whose inhibition
consistently elevates p53 activity in 3 different cell lines, 293, HCT116 and IMR-
90. This list is provided as Figure 4.

Homo sapiens calcium/calmodulin-dependent protein kinase (CaM
Homo sapiens CDC7 cell division cycle 7 (S. cerevisiae) (CDC7),
Homo sapiens choline kinase (CHK), mRNA.
Homo sapiens cyclin-dependent kinase (CDC2-like) 11 (CDK11),
mRNA.
Homo sapiens discoidin domain receptor family, member 1 (DDR1),

Homo sapiens discs, large homolog 1 (Drosophila) (DLG1), mRNA.
Homo sapiens discs, large homolog 2, chapsyn-110 (Drosophila)
Homo sapiens dual-specificity tyrosine-(Y)-phosphorylation
Homo sapiens fidgetin (FIGN), mRNA.
Homo sapiens glucokinase (hexokinase 4, maturity onset diabetes of
Homo sapiens IL2-inducible T-cell kinase (ITK), mRNA.
Homo sapiens KIAA0781 protein (KIAA0781), mRNA.
Homo sapiens KIAA0781 protein (KIAA0781), mRNA.
Homo sapiens KIAA0781 protein (KIAA0781), mRNA.
Homo sapiens KIAA1634 protein (KIAA1634), mRNA.
Homo sapiens LOC222383 (LOC222383), mRNA.
Homo sapiens membrane protein, palmitoylated 2 (MAGUK p55
subfamily
Homo sapiens membrane protein, palmitoylated 5 (MAGUK p55
subfamily
Homo sapiens microtubule associated serine/threonine kinase 3
Homo sapiens mitogen-activated protein kinase 6 (MAPK6), mRNA.
Homo sapiens mitogen-activated protein kinase kinase kinase 12
Homo sapiens mitogen-activated protein kinase kinase kinase kinase3
Homo sapiens muscle, skeletal, receptor tyrosine kinase (MUSK),
Homo sapiens mutL homolog 3 (E. coli) (MLH3), mRNA.
Homo sapiens myosin IIIB (MYO3B), mRNA.
Homo sapiens non-metastatic cells 1, protein (NM23A) expressed in
Homo sapiens non-metastatic cells 5, protein expressed in
Homo sapiens non-metastatic cells 5, protein expressed in
Homo sapiens phosphoinositide-3-kinase, class 3 (PIK3C3), mRNA.
Homo sapiens phosphoinositide-3-kinase, regulatory subunit 4, p150
Homo sapiens phosphorylase kinase, beta (PHKB), mRNA.
Homo sapiens protein kinase C, beta 1 (PRKCB1), mRNA.
Homo sapiens protein kinase C, beta 1 (PRKCB1), mRNA.
Homo sapiens ribosomal protein S6 kinase, 90kDa, polypeptide 2
Homo sapiens ribosomal protein S6 kinase, 90kDa, polypeptide 5
Homo sapiens serine/threonine kinase 16 (STK16), mRNA.
Homo sapiens serine/threonine kinase 38 (STK38), mRNA.
Homo sapiens serine/threonine kinase PSKH2 (PSKH2), mRNA.
Homo sapiens similar to adenylate kinase 1 (LOC200047), mRNA.
Homo sapiens similar to Ephrin type-A receptor 6 precursor
Homo sapiens similar to MAP/microtubule affinity-regulating kinase
Homo sapiens similar to myosin light chain kinase (MLCK)
Homo sapiens similar to Pyruvate kinase, M1 isozyme (Pyruvate
Homo sapiens tripartite motif-containing 33 (TRIM33), transcript
Homo sapiens tumor rejection antigen (gp96) 1 (TRA1), mRNA.
Homo sapiens UMP-CMP kinase (UMP-CMPK), mRNA.
Homo sapiens unc-51-like kinase 2 (C. elegans) (ULK2), mRNA.
Homo sapiens vaccinia related kinase 1 (VRK1), mRNA.
Homo sapiens v-ros UR2 sarcoma virus oncogene homolog 1 (avian)

Figure 4. Kinases whose inhibition activates p53. Positives from the p53 activation screen with the kinase shRNA library are listed. Cases where a name appears more than once indicates that multiple, independent shRNAs to the

same gene scored in the assay.    In some cases,  (e.g. PI3KC3 and p150) both a kinase and its regulator were identified.  All of these positives also activated p53 activity in HCT116 and IMR90 cells.

In depth studies of the class 3 PI3 kinase are proceeding.  These indicated that among the attributed activities of this protein, a defect in endocytosis might lead to p53 activation; however, additional genetic experiments will be required to validate this conclusion.

Highly parallel approaches

Since individual vectors contain unique barcodes that have been associated with each shRNA by sequence analysis, we can use a population-based approach to identify genes that interact with specific pathways.  In short, a population of cells is infected with the mixed library under conditions in which each construct is represented by multiple individual infected cells (~1000 cells/construct).  This population is subjected to a stress (genetic, environmental or chemical) while a control population remains untreated.  The barcodes representing individual vectors are then recovered from the populations by PCR.  The frequency change in each clone within the population can then be measured by competitive hybridization of the barcode material to DNA microarrays.  For those shRNAs that confer resistance to a treatment, we expect enrichment.  Conversely, for those barcodes that sensitize a cell to a particular treatment, we expect a reduction in frequency.

The overall approach is depicted schematically in Figure 5.

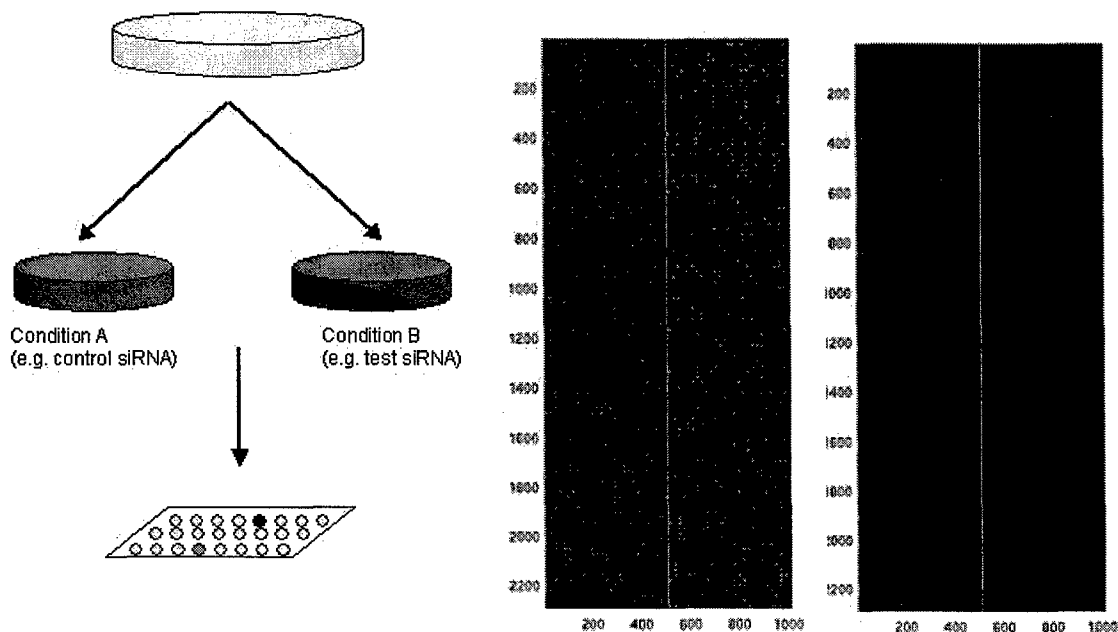# Highly parallel phenotypic screens using RNAi libraries



Figure 5  Highly parallel approaches toward synthetic lethal phenotypes.

Last year, we showed that one could assay barcode populations with a complexity of ~20,000 in mixed pools at time 0. However, as we analyzed later time points we saw tremendous amounts of noise. This made the barcode strategy unworkable as it stood. Over the past year, we have examined numerous conditions for stabilizing barcoded populations and have found that using pools of restricted complexity (presently 1000 hairpins per pool) that we can get reproducible results from parallel populations cultured for as much as 10 days. We are now using collections of cells engineered with 1400 kinase shRNAs to test for synthetic interactions between these kinase shRNAs and targeted therapeutics. Should these assays work, we can proceed to using siRNAs in place of drugs to search for bona fide synthetic lethal interactions.

**Key Research Accomplishments**

- Transformation of normal human cells in the absence of telomerase activation or other telomere maintenance strategies

- Discovery that short hairpin RNAs (shRNAs) can trigger stable gene silencing by RNAi in mammalian cells

- Discovery that shRNAs are effective in living animals

- Design of efficient delivery strategies for shRNA expression.

- Preparation of 80,000 shRNA expression vectors from the library

- Validation of the library in a screen for p53 activators

- Identification of 15 shRNAs that reproducibly activate p53 in multiple cell lines.

- Design of viable strategies for using barcodes to screen for phenotypes

## Reportable outcomes

Manuscripts

Seger, Y. R., Garcia-Cao, M., Piccinin, S., Lo Cunsolo, C., Doglioni, C., Blasco, M. A., Hannon, G. J. and Maestro, R. (2002) Transformation of normal human cells in the absence of telomerase activation. Cancer Cell 2: 401-413.

Silva, J. M., Mizuno, H., Brady, A., Lucito, R. and Hannon, G. J. (2004) RNAi microarrays : High-throughput loss-of-function genetics in mammalian cells. Proc. Natl. Acad. Sci., 101:6548-6552.

Paddison, P.P and Hannon, G. J. (2002) RNA interference : the new somatic cell genetics? Cancer Cell **2**:17-23

Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J. and Conklin, D.S. (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev. **16** : 948-958.

McCaffrey, A., Meuse, L., Pham, T. T., Conklin, D. S., Hannon, G. J. and Kay, M. A. RNA interference in adult mice. (2002) Nature **418** : 38-39.

Cleary, M., Kilian, K., Wang, Y., Bradshaw, J., Ge, W., Cavet, G., Paddison, P. J., Chang, K., Sheth, N., Leproust, E., Coffey, E. M., Burchard, J., Linsley, P. and Hannon, G. J. (2004) Production of complex libraries of defined nucleic acid sequences using highly parallel in situ oligonucleotide synthesis. Nature Methods, 1:241-248.

Reagents
- shRNA expression vectors
- A web site for shRNA and siRNA design (http://www.cshl.org/public/SCIENCE/hannon.html)

- shRNA delivery systems (retroviruses)
- shRNA libraries (codex.cshl.edu)

## Conclusions

The goal of this linked Idea and CDA award was to apply the genetic concept of synthetic lethality to the identification of new targets for breast cancer therapy. The overall goal has been to identify genes upon which cancer cells depend for survival but whose absence can be tolerated by normal cells. A series of approaches was proposed, but in many ways the program was a bit ahead of its time in terms of existing technological capabilities.

When this application was submitted in 1999, genetic approaches in mammalian cells were limited to the overexpression of cDNAs or the use of antisense RNAs to attenuate gene expression. RNAi was known as a phenomenon in C. elegans and it had been extended to Drosophila. However, the goal of using RNAi as a tool in mammalian cancer cells was a dream for the future. Over the funding period and partially with support from these linked awards, we have remade the landscape of possibility in human cancer genetics. Our work on the underlying biochemistry and genetics of RNAi in invertebrates revealed that known non-coding RNA loci fed into the RNAi pathway to regulate gene expression. These silencing triggers, now known generically as microRNAs, were found in an array of biological systems but most importantly for the purposes of the subject research programs, mammals. We seized upon this finding with the goal of permanently manipulating gene expression in a mammalian cell by redesigning these small RNAs, altering their specificity to regulate the expression of any protein coding gene, at will. Although our initial attempts succeeded in suppressing cancer-relevant gene expression both in cell lines and in intact animals, those tools were primitive. Over the past twelve months, we have created a second generation of silencing triggers that have improved performance. We are now able to design with a greater than 50% success rate artificial microRNAs that can strikingly repress target gene expression, even when present in a single copy in the genome of a target cell. With the support of a DOD innovator award, we have used these tools to create genome-wide libraries of shRNAs that now span 2/3 of all known and predicted genes in human and mouse cells with the library containing more than 100,000 sequence verified, individually arrayed constructs. The impact on the cancer community can be measured, even in the early going, by the fact that we have distributed more than 1,000,000 plasmids to investigators worldwide.

With the tools developed in the context of these linked idea and career development awards, we are now seeking to fulfill our original goals. As a start, we have carried out a pilot screen using only a set of shRNAs targeting kinases. We have found that several of these, when suppressed, activate the p53 pathway. Although we are only now delving into the biology of these individual pathways, several conclusions can already be drawn. First, each one of these kinases represent a potential therapeutic target. Present chemotherapeutic

strategies operate substantially through the use of DNA damaging agents to kill tumor cells through p53 activation. However, such drugs have potent side effects and may accelerate the formation of drug resistant clones through their mutagenic effects. An anti-kinase drug that engages the p53 pathway may still have a therapeutic effect on breast cancer, as do conventional therapies, without some of the possible side effects. A second outcome of these studies is the potential to link p53 to additional biological pathways. For example, we are currently pursuing studies of a class III PI3 kinase that indicates that p53 senses disruptions of the endocytic pathway – a link that had not previously been suspected. Pursuing the biology of even these early results could consume many student and postdoc lab-years. However, we are really searching for something more profound. Our hope is to use the tools that we have developed for the benefit of those that fail first line therapies. Toward this end, we have developed high throughput methods for measuring the response of a cell to suppression of gene expression in different contexts using microarrays. Using this approach we have recently begun searching for loci that affect the response of cells to a targeted therapeutic, Tarceva. We chose this as an initial screen because we have at least one positive control, but we hope to extend this paradigm once we have results from our first set of studies.

In all honesty, we did not achieve the ambitious goals of the original proposal. However, our striving toward these goals has had an impact on the field that is probably greater than if we had succeeded in a less ambitious project.